

# Antitrichomonal antibody in the vaginal secretions of women infected with *T. vaginalis*

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Trichomoniasis, a common and sometimes distressing condition in women, is caused by infection of the genitourinary tract by the pathogenic protozoan *Trichomonas vaginalis*. Men may also be infected, although frequently without showing any symptoms, and most cases of trichomoniasis are sexually transmitted (Whittington, 1957; Catterall and Nicol, 1960). In women, the parasite may be found in the vagina, urethra, bladder, and Skene's ducts, and occasionally in Bartholin's glands, but the organisms do not as a rule ascend into the uterine cavity or Fallopian tubes (Catterall, 1972), and parasitaemia does not occur. Despite the existence of effective chemotherapeutic agents, the incidence of trichomoniasis is increasing, and this has led to renewed interest in the immunological response of the host to this infection. The sera of chronically infected women have been shown to contain low titres of antibodies detectable by indirect immunofluorescence (Kramar and Kučera, 1966) and indirect haemagglutination (Hoffmann, 1966), and also higher titres of complement-fixing and agglutinating antibodies (results from a large number of papers by the Estonian group of Teras (1962) and his colleagues are summarized by Honigberg (1972)); however, these antibodies appear too slowly after infection to be of much diagnostic value.

The immune response to antigens at a mucosal surface appears to consist of an initial, localized production of secretory IgA, followed later by a more widespread IgG response (Brandtzaeg, 1973). It was therefore decided to look for a local rather than a systemic antibody response to *T. vaginalis*, because it seemed likely that the former would appear more rapidly than the latter and local antibodies might be the only host responses to such a non-invasive parasite.

## Material and methods

### COLLECTION AND HANDLING OF SPECIMENS

Specimens of vaginal secretion were collected, using a method similar to that of Robertson, Lumsden, Fraser, Hosie, and Moore (1969), from 508 female patients attending James Pringle House (the sexually-transmitted diseases clinic of the Middlesex Hospital) for the first time. Polyester sponge cubes (Campbell Brushes Ltd., Bellingdon Road, Chesham, Bucks) were cut into pieces approximately  $1 \times 1 \times 4$  cm. and these were autoclaved individually in 5-ml. screw-capped bottles. After routine examination the sponge was inserted into the vagina using forceps, allowed to absorb the fluid present, and returned to the bottle. The bottles were collected from the clinic twice a day; any specimens taken after the second collection were discarded and no specimens were kept for more than 4 hrs before being examined. Bottles awaiting collection were kept at room temperature. Preliminary experiments had shown that in these conditions cultured *T. vaginalis* suspended in normal saline and absorbed in sponge remained viable for at least 5 hrs.

To recover the secretions 1 ml. sterile normal saline was added to each sponge and the bottle was attached to the top socket of a sterile Hemmings filter holder. No filter pad was used. A sterile 5-ml. bottle was attached to the bottom socket and the assembled filter holder was centrifuged at 250 G. for 10 min. After centrifugation the lower bottle contained a clear supernatant (which was withdrawn and stored at  $-20^{\circ}\text{C}$ ) and a pellet. The pellet was re-suspended in 0.2 ml. culture medium and one drop was used to make a wet film; the remainder was used to inoculate 15 ml. culture medium in a 15-ml. screw-capped bottle (see below for details of medium). Minimum air space in the bottle, and non-inhibitory white rubber wads are necessary to obtain growth from a small inoculum (Lumsden, Robertson, and McNeillage, 1966). Wet films were examined under phase contrast at  $400\times$  total magnification and the presence of *T. vaginalis*, epithelial cells, and pus cells was noted and scored on arbitrary scales. (These scales were: For *T. vaginalis*: 0 = no organisms seen; 1 = less than one organism per field; 2 = one to two organisms per field; 3 = three to four organisms per field; 4 = more than four organisms per field. For epithelial and pus cells — 1 = a few scattered cells; 2 = many cells; 3 = cells almost confluent.

All the slides were scored by the same observer.

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Cultures were examined daily for colour change indicating the production of acid, and microscopically for the presence of *T. vaginalis*. Cultures still negative after 7 days' incubation were discarded. Most of the strains isolated were cryopreserved in liquid nitrogen by the method of Lumsden and others (1966) until required for antibody assay. To avoid antigenic variation, strains were always cryopreserved from the first culture obtained.

#### CULTURE MEDIUM

Organisms were grown in the medium of Lumsden and others (1966) modified by the replacement of liver digest by Neutralized Liver Digest (Oxoid), and by altering the pH of the buffer component to 6.8. The final pH of the medium was checked and adjusted to 6.8 if necessary. This medium contains penicillin G (1,000 units/ml.), and streptomycin sulphate (1 mg./ml.); nystatin (250 µg./ml.) was added to the medium only for the primary isolation of *T. vaginalis* from vaginal secretions.

#### CLINICAL DETAILS

After the completion of the antibody assays the patients' records at James Pringle House were examined and the following information recorded: clinical diagnosis, duration of symptoms, and use of oral contraceptives.

#### <sup>125</sup>I-LABELLED ANTIHUMAN IMMUNOGLOBULINS

Antiserum from sheep immunized with human serum IgA was purchased from Wellcome Reagents Ltd. This is stated by the manufacturers to be monospecific by immunoelectrophoresis and immunodiffusion, but absolute specificity was not important for our purposes and was not checked. Immunoglobulin was prepared from the serum by precipitating three times with 35 per cent. saturated ammonium sulphate at pH 6.5 (Herbert, Pelham, and Pittman, 1973), and the final product was freed from ammonium sulphate by dialysis against phosphate-buffered normal saline (0.01M sodium phosphate pH 7.2) (PBS). The protein concentration was measured by the Lowry, Rosebrough, Farr, and Randall (1951) modification of the Folin-Ciocalteu method, using crystalline bovine serum albumin as standard, adjusted to 10 mg./ml. and the solution was stored at -20°C until required.

Iodination of the antihuman IgA globulin was by the method of McConahey and Dixon (1966), using 200 µl. protein solution (10 mg./ml.) and 1 mCi carrier-free iodine -125 (Code IMS 30; Radiochemical Centre, Amersham). Unbound iodine was removed by dialysis against PBS and the final volume of protein solution made up to 5 ml. Working dilutions were made and stored at -20°C. <sup>125</sup>I-labelled antihuman IgG was prepared from sheep antihuman IgG serum (Wellcome) by the same method; the serum was not rendered heavy chain specific and cross-reacted with both IgA and IgM.

#### ANTIBODY ASSAY

The contents of a single 25-µl. capillary tube of cryopreserved *T. vaginalis* were inoculated into 15 ml. of culture medium and were grown at 37°C for 3 to 5 days until a haemocytometer count of  $1-2 \times 10^6$  organisms/ml. was reached. Organisms were harvested by gentle centri-

fugation (250 G. for 15 min.) washed twice with PBS, and re-suspended in PBS containing 1 per cent. v/v inactivated calf serum (Wellcome) and 0.02 per cent. w/v sodium azide (PBSSA), to give a final count of  $4-8 \times 10^6$  organisms/ml.

Vaginal secretions to be tested for antibody were centrifuged at 3,300 G. for 30 min. to remove any suspended material. Each patient's secretion was tested against the organisms isolated from her; secretations from one or more non-infected women were tested against the same organisms in parallel.

The assay was carried out in stoppered 2-ml. polystyrene tubes (Code LP/3; Luckham Ltd., Victoria Gardens, Burgess Hill, Sussex) which had been soaked before use for at least 24 hrs in 0.1M sodium bicarbonate containing 3 per cent v/v inactivated calf serum. Tubes were washed five times in PBSSA and the assay then set up using 200 µl. *T. vaginalis* suspension and 100 µl. of secretion. Controls were set up with organisms alone, secretion alone, and PBSSA alone. The contents of the tubes were mixed well and kept at room temperature for 1 hr before being centrifuged at 2500 G. for 15 min. The supernatants were discarded and the pellets washed three times with 1 ml. PBSSA. 25 µl. PBSSA followed by 25 µl. diluted <sup>125</sup>I-labelled antihuman IgA was then added to each tube; a single batch of anti-IgA was used for all assays, initially diluted 1/20 but later at 1/10. After mixing, the tubes were kept for a further hour after which they were centrifuged and the pellets again washed three times. Finally 500 µl. PBSSA was added to all tubes, and the contents were mixed well and transferred to clean tubes for counting. Counts due to bound antibody were obtained by subtracting from the crude figures counts bound by organisms, secretions, and the tube separately, and were then corrected for radioactive decay. Appropriate controls showed that organisms and antihuman IgA were present in at least two-fold excess over that required to bind all the antibody present in even the most active secretions.

#### Results

Specimens of vaginal secretion were obtained from 508 women; *Trichomonas vaginalis* was cultured from 42 of these (8.3 per cent.). In 32 cases the organisms were also visible in the wet preparation. All cases with organisms present in the wet film showed positive results on culture.

Secretions from 29 of the infected women and from 19 non-infected women were assayed for antibody against the organism with which they had been infected using <sup>125</sup>I-labelled antihuman IgA. Assays were performed twice, and the average of the two figures taken.

The average number of counts bound when secretions from infected women ('patients') were assayed, was 13,500; this was higher than the average for women not currently infected ('controls') which was only 3,970. In both groups two populations were defined (Figure), those with counts below 1,000 ('no antibody') and those with counts above 1,000 ('with antibody'). Amongst the patients, 22 out of 29 (76%)

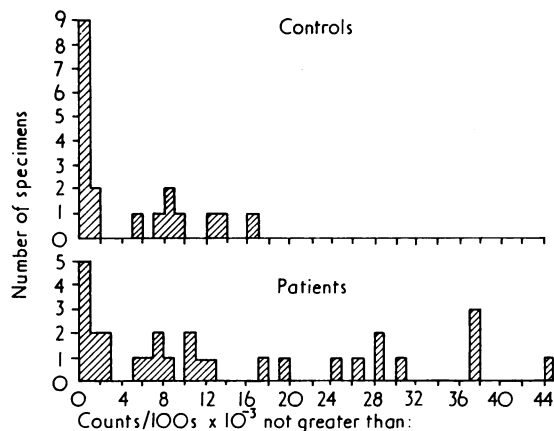


FIGURE Findings in patients and controls

had secretions with antibody; the corresponding figure for the controls was 8 out of 19 (42 per cent.). This difference is statistically significant ( $\chi^2 = 5.58$ ;  $0.05 > P > 0.01$ ). Moreover, not only were there fewer controls than patients with antibody but the amount of antibody present was lower; for patients with antibody the average number of counts was 17,900, but for the corresponding group amongst the controls it was 9,280.

As noted above, wet films were scored on an arbitrary scale for number of epithelial and pus cells. No significant differences were found in the epithelial cell score between patients and controls; although patients had a higher average score for pus cells than controls (2.4 and 1.7 respectively), there was no significant difference among either patients or controls between the average pus cell scores for those with and those without antibody. Similarly, there was no significant difference in the use of oral contraceptives either between patients (15/28; 54 per cent.) and controls (9/19; 47 per cent.) or, within these groups, between those secreting and not secreting antibody.

In an attempt to understand the existence of a group of women who, although infected with *Trichomonas vaginalis*, were not producing detectable antibody against the parasite, we examined the duration of any symptoms suggestive of infection up to the time the specimen was collected. Excluding two patients (Nos 2184 and 2551) whose symptoms had persisted for an exceptionally long period, the average duration of symptoms for remaining patients was 23 days; the averages of those with and without antibody were 21 and 26 days respectively. Neither these figures nor those for the presence of additional sexually-transmitted diseases are insignificantly different; amongst the patients eleven out of 22 of those with antibody and three out of seven of those with no antibody had multiple infections.

There was, however, some evidence of a negative correlation between the presence of antibody and the numbers of organisms in the vagina. Wet films scored for the numbers of trichomonads showed a mean score for patients with antibody of 1.9 compared with 2.9 for those without antibody. Moreover, ten of the patients in this study were diagnosed by culture only, and in these cases the number of trichomonads may be presumed to have been very low; seven of these secretions could be tested for antibody and all were found to be positive.

Only two women admitted to an earlier infection with *T. vaginalis*; one was a patient with antibody and the other had no antibody.

Serum was obtained from eighteen additional women (none of whose secretions had been included in the main assay) and assayed for antitrichomonal antibody. The same method was used except that the anti-IgA was replaced by <sup>125</sup>I-labelled antihuman immunoglobulin prepared against human IgG but also cross-reacting with IgA and IgM. Nine sera were from patients and nine from controls; no antitrichomonal antibody was detected in any of them.

## Discussion

The primary aim of this work was to look for evidence of antitrichomonal antibodies in the vaginal secretions of women infected with *T. vaginalis*; two previous attempts to find secreted antibody in such patients were not successful, although the numbers involved were small. Rom and Thierry (1958) using direct agglutination found no evidence of antibody in three patients, and Hosie (1971) using indirect immunofluorescence found no positive results in secretions from ten patients. It was therefore clear that if antibody was present it would be in low concentration and the method used was chosen because we felt that it would be both sensitive and objective. We tested each patient's secretion against her own trichomonads since the existence of serotypes amongst *T. vaginalis* isolates has been suggested (Teras, 1962) and the use of homologous organisms should eliminate the risk of some antibody-containing secretions being missed. Two problems encountered during the performance of the assay were the very high counts found in tubes which contained either secretion alone or organisms alone. The counts bound by secretion alone were attributed to binding of anti-IgA to human immunoglobulin from the secretion which had been absorbed on to the walls of the assay tubes; the problem was solved by pre-coating the tubes with heterologous (calf) serum protein, by using 1 per cent. calf serum for all dilutions and washings, and by transferring the washed organisms to clean tubes for counting. No means, however, could be found of reducing the large number of counts bound to organisms alone; the number of

counts bound was not significantly reduced by washing up to ten times. It is known that, when *T. vaginalis* is grown in serum containing media, the washed organisms are contaminated with serum proteins (Oelerich and Mannweiler, 1968); we therefore wondered if the counts were being incorporated by binding of antihuman IgA to calf immunoglobulin absorbed on to the organisms from the culture medium. This, however, seems unlikely for several reasons:

- (1) As expected the antihuman IgA gave no detectable precipitation lines when tested by double diffusion against calf serum or whole culture medium;
- (2) The counts due to *T. vaginalis* alone were not significantly reduced when the organisms were grown in medium in which normal calf serum was replaced by foetal calf serum.
- (3) Pretreatment of the washed organisms with trypsin (0.4 per cent. crystalline trypsin in PBS pH 7.4 at 37°C for 45 min.), which might have been expected to remove absorbed immunoglobulin, had no effect on the number of counts bound.

We also wondered if the incorporation of radioactivity was due to pinocytosis by the still living trichomonads; however, there was no significant difference between the number of counts bound by unkilld and formalin-killd organisms. Thus we do not at present know why so much radioactivity is incorporated in the absence of antibody; the need to make a large correction for this activity necessarily affects the accuracy of the results. In addition, the recovery of liquid from the sponges was not reproducible and it was consequently not possible to estimate the dilution which had occurred during collection of the specimens. It thus seems preferable to discuss the results in terms of the presence or absence of antibody rather than in terms of the amount actually present.

Antibodies apparently directed against *T. vaginalis* were found in the vaginal secretions of both patients and controls, but in a significantly higher proportion of the former. The reasons why some patients failed to produce antibody, while some of the controls did, are not clear. There was no significant difference amongst patients with and without antibody in duration of symptoms, degree of inflammation (as measured by epithelial and pus cells in secretions), or the presence of additional sexually-transmitted diseases. Among the non-infected controls, we could find no correlation between antibody production and degree of inflammation or the nature, if any, of their present venereal infections. There was no evidence that any of these women had been previously infected with *T. vaginalis*, and we can offer no explanation for the presence of antibody in their vaginal secretions. It has been suggested (Chipperfield, personal com-

munication) that use of oral contraceptives has an effect on the immunoglobulin content of vaginal secretions but no correlation could be found between antibody in secretions and the use of oral contraceptives.

Similar secreted antibody, identified as locally produced IgA, has recently been reported in the urethral exudate of about 80 per cent. of men with uncomplicated gonorrhoea (Kearns, O'Reilly, Lee, and Welch, 1973) and in an unstated number of similarly infected women (O'Reilly: quoted by Kearns and others, 1973). The fact that we could not detect antibody in any of the small number of sera tested suggests that the antibodies reported here were also locally produced; an increase in the number of IgA and IgM secreting cells in vaginal biopsies from women infected with *T. vaginalis* has already been reported (Chipperfield and Evans, 1972).

Untreated trichomoniasis in women tends to subside into a chronic low-grade infection with periodic recrudescence of symptoms; complete elimination of the parasite does not usually occur. Clearly, therefore, the antibodies we have found are not capable of eliminating the infection and this immune response does not prevent re-infection, for repeated attacks of trichomoniasis are common. It is too early to say what significance, if any, to attach to the apparent association of low parasite counts with the presence of antibody.

Infection with *T. vaginalis* is much more difficult to detect in men than in women; we are now examining secretions from male contacts; if antitrichomonal antibody can be detected in these secretions and if the overlap between patients and controls can be reduced, such assays may be of considerable value in contact tracing, and in decisions concerning the treatment of symptomless male contacts of infected women.

### Summary

Vaginal secretions from 508 women were examined for evidence of infection by *Trichomonas vaginalis*, and for antibodies directed against this organism; 42 women (8.3 per cent.) were found to be infected.

Secretions from 29 of these women were assayed and antibody apparently directed against *T. vaginalis* was found in 22 (76 per cent.) of them. Eight out of nineteen secretions (42 per cent.) from apparently uninfected women also contained antibody. Amongst the infected women, no correlation could be found between the presence or absence of antibody and the degree of inflammation, duration of symptoms, use of oral contraceptives, or additional sexually-transmitted disease; there was, however, a slight suggestion that low parasite counts in the vaginal secretions were associated with the presence of antitrichomonal antibody.

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